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In vitro culturing of yellow starthistle (*Centaurea solstitialis*) for screening biological control agents

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Abstract

In an effort to design a bioassay to screen for biocontrol pathogens and their toxins, procedures were developed to produce calli of yellow starthistle (YST), *Centaurea solstitialis* (Asteraceae), on solid and liquid media. Three Murashige and Skoog (MS) media with different hormone additives were compared for their effects on the growth of the YST calli. The most effective medium was made of MS salts supplemented with two cytokinins and a low rate of auxin. The growth of the calli slowed when cytokinins were omitted. Similar results were observed in a bioassay setup to compare the reactions of the YST calli with those of detached leaves exposed to potential toxins in the culture filtrates of test fungi. After exposure for 96 h to different concentrations of culture filtrates from three *Alternaria alternata* isolates, the calli exposed to 100 and 50% concentrations of the filtrates were significantly more damaged than other concentrations tested, based on a visual rating scale. No differences were observed in the damage ratings among the *A. alternata* isolates tested. An additional bioassay was setup to determine if calli could be used to screen pathogenic fungi. After 48 h, a distinction could be made in a visual rating between calli treated with spores of a pathogen, *Phoma exigua*, and a non-pathogen, *Penicillium* sp., confirming that YST calli can be used to screen potential pathogenic fungi against YST.

Keywords: *Alternaria alternata*; Biological control; Callus; *Centaurea solstitialis*; *Phoma exigua*; Tissue culture; Toxins; Yellow starthistle

1. Introduction

Yellow starthistle (*Centaurea solstitialis* L.), is a noxious weed in the United States that displaces native plant communities and reduces plant diversity. Hartmans et al. (1997) consider it the most serious weed problem in northern Idaho rangelands and it is estimated to infest between 5 and 8 million hectares in California alone (DiTomaso et al., 1999). Millions of dollars are spent each year to manage yellow starthistle on rangelands, recreational areas, and highways. Yellow starthistle can be managed through the use of chemicals, grazing, and fire (DiTomaso et al., 1999; Thomsen et al., 1993). Postemergence herbicides are effective, but this method is expensive, laborious, and herbicide-resistant populations have developed (Callihan et al., 1990; Northam and Callihan, 1989, 1991). Yellow starthistle is a prime target for biological control because the control strategies mentioned above have been inadequate due to the large size of infestations and the economic and environmental costs of chemical control.

Search for biological control agents for yellow starthistle has resulted in the introduction of six insects into the United States (Rees et al., 1995; Turner et al., 1995). All but one of these insects have established and spread, but control has been limited. Plant pathogens also have been studied as potential biological control agents. However, most of the indigenous pathogens found in the United States are not host specific (Klisiewicz, 1986; Woods and Fogle, 1998). Exploration in the native range of yellow starthistle has yielded potentially new agents (Bruckart and Dowler, 1986; Widmer et al., 2002). One fungus discovered in Turkey, *Puccinia jaceae* Otth, was found to be host specific and has been released in the United States (Bruckart, 1989). Additional agents are being sought, but the work is hampered by the lack of ready availability of clean plant material for testing

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and a rapid method for screening and testing potential agents. To overcome these limitations, we have examined tissue culture as a means of rapid screening.

In vitro culture of weeds has many potential applications. Herbicide screening and resistance can be conducted using callus tissue. Johnsongrass (*Sorghum halepense* L.) callus was used to evaluate variation in glyphosate tolerance (Kintzios et al., 1999). Cocklebur (*Xanthium strumarium* L.) callus was used to compare herbicide-resistant and susceptible tissues (Ellis and Camper, 1995). Production of host-plant tissue by in vitro methods to screen and scale-up production of biological control agents is another important application. A shoot-culture system was used to rear the nematode *Subanguina picridis* (Kirj.) Brezeski, a candidate biocontrol agent for Russian knapweed (*Acroptilon repens*) (Ou and Watson, 1993). Successful culturing of the knapweed allowed for mass rearing of the nematode in a limited space and without the time-intensive plant maintenance. Callus cells of *Galium spurium* L. were used also to produce *Plectosporium tabacinum* (van Beyma) Palm et al., which is being tested as a biocontrol agent against this weed (Zhang et al., 2001). The use of tissue culture allows for a rapid and inexpensive method to screen microbial agents and has been found to be representative of results obtained in whole-plant experiments studying different aspects (Souissi and Kremer, 1998). Tissue culture of *Rubus* sp. was set up to screen for phytotoxins from fungal pathogens (Hollmann et al., 2002), and leafy spurge (*Euphorbia esula* L.) callus to screen and study rhizobacterial isolates (Souissi and Kremer, 1998; Souissi et al., 1997). Correlations between efficacy of phytotoxins at the tissue-culture level and the whole-plant level have been made before (Hogan and Manners, 1990; Song et al., 1994). Souissi and Kremer (1998) quantitatively correlated cell viability and fresh weight of callus with a visual rating of callus upon exposure to bacteria.

The objective of this study was to develop a procedure to produce callus, cell-suspension cultures, and whole plants from cell cultures of yellow starthistle. The callus was tested for its reaction against fungal toxins, a pathogenic fungus, and a nonpathogenic fungus to determine its potential as a screening tool. This technique should enable rapid screening of biological control agents and culturing of microorganisms and insects on yellow starthistle.

2. Materials and methods

2.1. Plant material

Yellow starthistle seeds were germinated on moist filter paper in a plastic petri plate at 23 °C under artificial light (16 h light/8 h dark). After 5 days, the seedlings were transplanted to a commercially available organic soil and maintained in a growth chamber at 23 °C under artificial light (16 h light/8 h dark). The plants were watered as needed.

2.2. Callus production

A modified half-strength Murashige and Skoog (1/2MS) medium was prepared as described (Murashige and Skoog, 1962). The modified stock solution of macro-elements consisted of 825 mg NH_4NO_3, 950 mg KNO_3, 220 mg $CaCl_2 \cdot 2H_2O$, 185 mg $MgSO_4 \cdot 7H_2O$, and 85 mg KH_2PO_4 in 1 liter of distilled water. A stock solution of micro-elements was prepared by adding 0.83 mg KI, 6.2 mg H_3BO_3, 16.9 mg $MnSO_4 \cdot H_2O$, 8.6 mg $ZnSO_4 \cdot 7H_2O$, 0.25 mg $Na_2MoO_4 \cdot 2H_2O$, 0.025 mg $CuSO_4 \cdot 5H_2O$, and 0.025 mg $CoCl_2 \cdot 6H_2O$ to 1 liter of distilled water. Murashige and Skoog vitamin mixture was purchased and prepared to make a 1000× stock solution (Duchefa Biochemie BV, Haarlem, The Netherlands). The medium was prepared by combining 50 ml of the macro-elements stock solution, 5 ml of the micro-elements stock solution, 1 ml of the vitamin mixture stock solution, 36.7 mg of FeNa · EDTA, and 30 g of sucrose to distilled water for a final volume of 1 liter. The pH was adjusted to 6.0 and 8 g of phyto agar was added. The medium was autoclaved and dispensed at approximately 25 ml into sterile 125-ml baby-food jars. Young rosette leaves (approximately 1-month-old) of yellow starthistle were removed at the base of the plant, and leaf sections (approximately 1 cm in diameter) were surface-sterilized for 30 s in 75% ethanol plus 2 or 3 drops of Tween 20 (Sigma–Aldrich Chimie, Saint Quentin Fallavier, France) and transferred to 0.5% calcium hypochlorite. After 20 min, the leaves were rinsed for 10 min in sterile distilled water and then twice for 5 min in sterile distilled water. Sections were placed into the baby-food jars containing 1/2MS medium. The jars were placed in an incubator at 26 °C with 16 h of fluorescent light. After callus tissue formed, the calli were broken off and transferred every 3 weeks to fresh 1/2MS medium.

2.3. Cell-suspension culture

A MS liquid medium was prepared according to Murashige and Skoog (1962); the 1/2MS medium was prepared as described above without the addition of agar. The medium was supplemented with 4 mg per liter of 2,4-dichlorophenoxy acetic acid (2,4-D; Duchefa Biochemie BV) to initiate friable calli. Yellow starthistle calli produced above were placed in 100 ml of autoclaved liquid medium and placed on an orbital shaker at 26 °C, 150 rpm with ambient light. After 2 weeks, the liquid medium plus 2,4-D is removed after sedimentation of the calli, and replaced with 100 ml of sterile,

liquid, 1/2MS medium without any growth regulator. After 2 weeks on the orbital shaker under the same conditions, the cells were transferred to solid 1/2MS medium for calli production.

2.4. Callus growth characterization

The growth of yellow starthistle (YST) callus on three previously described media was compared to determine an optimal growth medium. All chemicals were purchased from Duchefa unless otherwise stated. Medium MS-CAL (Pasqualetto and Dunn, 1989) was prepared by supplementing Murashige and Skoog salts with 25 mg NaH_2PO_4, 20 mg FeNa · EDTA, 100 mg myo-inositol, 0.4 mg thiamine–HCl, 40 mg adenine sulfate, 0.1 mg 1-naphthalene-acetic acid (NAA), 0.5 mg 6-furfurylaminopurine (kinetin), 0.5 mg *N*-(-2-isopentenyl)-adenine (2iP), 30 g sucrose, and 8 g phyto agar per liter of medium. Medium MS-CAR (Gamborg and Phillips, 1995) was prepared by supplementing Murashige and Skoog salts with Murashige and Skoog vitamin mixture, 20 mg FeNa · EDTA, 2 mg 2,4-D, 30 g sucrose, and 8 g phyto agar per liter of medium. Medium MS-T1 (Gamborg and Phillips, 1995) was prepared by using Murashige and Skoog salts supplemented with Murashige and Skoog vitamin mixture, 20 mg FeNa · EDTA, 2 mg NAA, 0.2 mg 2iP, 2 mg 2, 4-D, 30 g sucrose, and 8 g phyto agar per liter of medium. The pH was adjusted to 5.8 prior to adding the agar and autoclaved for 30 min at 121 °C. Molten media were poured into sterile petri plates (90-mm diameter) and allowed to solidify.

Seven pieces of fresh calli were transferred to three plates of each of the media. The diameter of each callus was measured and the plates were placed in an incubator at 26 °C with a 16 h photoperiod under fluorescent light. After 12 days, the diameter of the calli was measured twice a week for 4 weeks. The average growth of the calli was compared over time using a SAS (SAS Institute, Cary, NC; v. 8.02) procedure for repeated measures analysis (PROC GLM).

2.5. Effect of fungi on callus

Phoma exigua Desmaz. and a *Penicillium* sp. were isolated from *C. solstitialis* plants in France and maintained on half-strength potato dextrose agar (1/2PDA). Conidia were produced by placing an actively growing plug on 1/2PDA and placing the plate under UV light. Spores were harvested by adding distilled water containing Tween 20 (0.25% v/v) on the agar surface and scraping with a rubber policeman. The concentration of spores was counted with the aid of a hemacytometer.

A healthy yellow starthistle callus piece grown for 2 weeks on solid 1/2MS was placed in each well of a 24-well, sterile, plastic microtiter plate that contained 0.5 ml of liquid 1/2MS plus 0.25% Tween 20. Spores of *P. exigua* and *Penicillium* sp. were added into wells for a final concentration of 1.0×10^6 conidia per ml (four repetitions per concentration). A control was set-up in the same manner with only liquid 1/2MS plus 0.25% Tween 20. The plate was placed in a 26 °C incubator and the calli were rated after 24, 48, and 96 h on a modified scale of Souissi and Kremer (1998) of 1 (green, healthy callus) to 5 (complete browning and disintegration of the callus). Ratings of 2–4 meant slight discoloration of the callus, some browning of the callus, and complete browning of the callus, respectively. The callus ratings were averaged within each time period and subjected to analysis of variance using GLM.

2.6. Effect of fungal toxins on calli

Three isolates (labeled YST3, YST4, and YST6) of *Alternaria alternata* (Fr.:Fr.) Keissl. were isolated from leaf spots on *C. solstitialis* leaves collected in France. Cultures were maintained on 1/2PDA. For production of *Alternaria* toxins, the culture was grown on a medium described by Maiero et al. (1991), which contained 1 g KH_2PO_4, 0.5 g $MgSO_4$, 6 g casein hydrolysate, 100 g sucrose, 1 mg $FeSO_4$, 0.15 mg $CuSO_4$, 0.1 mg $ZnSO_4$, and 0.1 mg Na_2MoO_4 per liter of distilled water. The medium was adjusted to pH 4.9 with 0.1 M HCl and autoclaved. Five mycelial plugs (2-mm diameter) from the edge of an actively growing colony of the tested *A. alternata* were added to 100 ml of the cooled medium. The cultures were incubated at room temperature in the dark for 6 weeks under stationary conditions. Culture filtrates were prepared by pouring the medium through several layers of cheesecloth. The filtrate was sterilized by passing through a 0.2-μm filter and stored at 4 °C. Combinations of sterile nutrient broth and *Alternaria*-filtrate were added to a sterile, 24-well, plastic microtiter plate to have 2 ml of final filtrate concentrations of 100, 50, 25, 10, 1, and 0%. There were six repetitions per treatment. A green, freshly transferred piece of callus, approximately 5 mm in diameter, was placed in each microwell. The plate was covered and placed in an incubator at 26 °C. After 96 h, the calli were rated on the same scale described above. The callus ratings were averaged within concentration and subjected to analysis of variance using GLM. The effect of the filtrate concentration on each *A. alternata* isolate was subjected to regression analysis. The experiment was repeated once.

To verify the toxicity of the filtrate on yellow starthistle plants, rosette leaves were removed and placed in a petri plate containing moistened filter paper. A drop of the culture filtrate was placed on each of the leaves, including a water control. The plates were placed in a growth chamber at 25 °C and the presence of necrotic symptoms was noted over time. The leaves were rated after 24, 48, and 96 h on a scale of 1 (no symptoms) to

5 (disintegration of the tissue). Ratings of 2–4 meant yellowing of the tissue, some browning of the tissue, and complete browning of the tissue, respectively.

3. Results and discussion

Development of yellow starthistle callus can be important in studying the plant and for screening potential biological control agents. Once callus was produced it was easy to maintain and propagate it. The cell-suspension method produced an abundance of viable cells that produced calli large enough to utilize after approximately 2 months.

Callus growth (= diameter) after 4 weeks on MS-CAL medium was superior than on the other two media tested ($P < 0.0001$) (Fig. 1). There was a significant effect on the callus diameter from the growth media, sampling time, and the interaction between the media and sampling time (Table 1). Growth of calli over time on MS-CAL was greater than on MS-CAR ($P = 0.0024$) and MS-T1 ($P = 0.0052$). This growth difference was observed within the first 2 weeks. After the third week, there was a clear difference between all three growth media. The growth curves of the calli started to level off for the calli grown on MS-CAR and MS-T1. MS-CAL is a balanced medium containing two cytokinins and a low rate of auxin, while MS-CAR and MS-T1 lack cytokinin. MS-T1 is supplemented with two forms of auxins, NAA and 2,4-D, that do not appear to add any benefit without the kinetin.

The culture filtrates of the *A. alternata* isolates tested showed obvious necrotic lesions on detached leaves of yellow starthistle after 24 h (Table 2). Within 24 h, calli exposed to the full-strength (100%) culture filtrate turned light brown in color, which related to an average rating of 3.4 compared to a rating of 1.0 for the control (0%). There was no difference on callus rating among the three isolates of *A. alternata*; however, the concentration of filtrate did affect the callus rating (Table 3), which was verified by regression analysis ($P < 0.001$). When the data from all three *A. alternata* isolates were combined within concentration, calli exposed to 100 and 50% strength of the filtrates had a rating of 4.2 and 4.1, respectively, after 96 h. In contrast, calli exposed to 1 and 0% filtrate concentrations remained green and healthy resulting in ratings of 1.3 and 1.0, respectively. The rating values of the calli in relation to the isolate and concentration are shown in Fig. 2. Since there was no difference in toxicity between the different isolates ($P = 0.7873$), no attempt was made to compare or quantify the toxin compounds between the isolates.

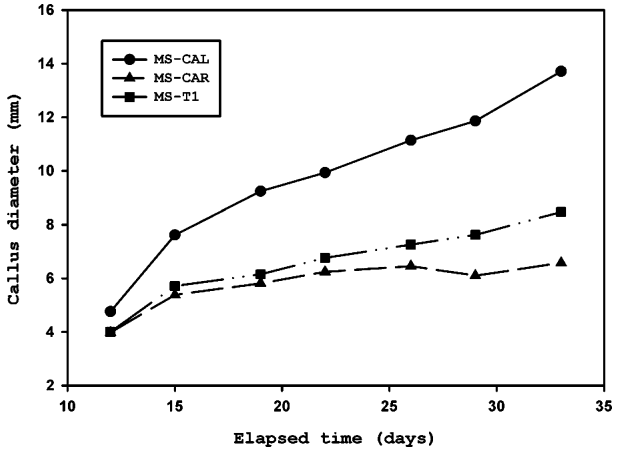

Fig. 1. The effect of different solid media on the growth of *Centaurea solstitialis* calli over time.

Table 1
Results of repeated-measures analysis of *Centaurea solstitialis* callus growth using a General Linear Model

Effect	*df*	Callus growth	
		F value	Pr > *F*
Medium (M)	2	491.99	<0.0001
Time (T)	6	141.59	<0.0001
M × T	12	19.10	<0.0001
Residual	411		

Table 2
Average ratings of detached leaves and callus of *Centaurea solstitialis* after 96 h of exposure to *Alternaria alternata* culture filtrates at full strength

Time (h)	Detached leaves[a]		Callus[b]	
	Control[c]	Treated	Control	Treated
24	1.0 ± 0.0	2.7 ± 0.7	1.0 ± 0.0	3.4 ± 0.5
48	1.0 ± 0.0	3.2 ± 0.4	1.0 ± 0.0	3.9 ± 0.4
96	1.0 ± 0.0	4.3 ± 0.8	1.0 ± 0.0	4.2 ± 0.4

[a] Average rating of *C. solstitialis* detached leaf at point of inoculation over time based on a scale of 1 (no effect) to 5 (complete disintegration of the leaf). Ratings of 2–4 mean yellowing of the leaf, some browning of the leaf, and complete browning of the leaf, respectively.

[b] Average rating of *C. solstitialis* calli over time based on a scale of 1 (green, healthy callus) to 5 (complete browning and disintegration of the callus). Ratings of 2–4 mean slight discoloration of the callus, some browning of the callus, and complete browning of the callus, respectively.

[c] Control detached leaves and calli are exposed to sterile distilled water and nutrient broth, respectively.

Table 3
Analysis of variance of ratings of *Centaurea solstitialis* calli after 96 h of exposure to culture filtrates of *Alternaria alternata*

Source	*df*	*F* value	Pr > *F*
Isolate	2	0.24	0.7873
Concentration	5	126.47	<0.0001
Residual	90		

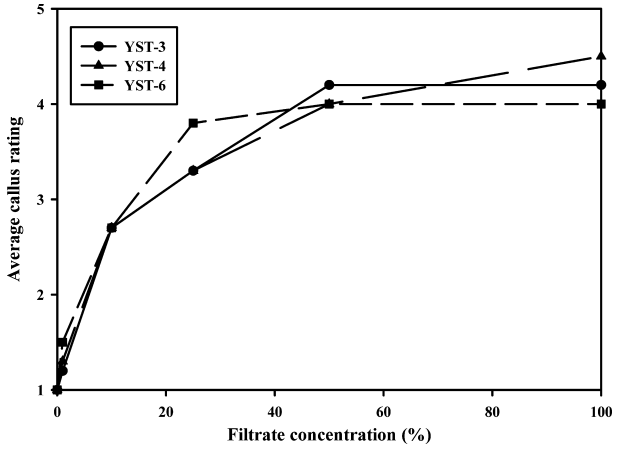

Fig. 2. Impact of toxin concentrations from three isolates of *Alternaria alternata* on *Centaurea solstitialis* calli. The average rating of *C. solstitialis* calli after 96 h based on a scale of 1 (green, healthy callus) to 5 (complete browning and disintegration of the callus). Ratings of 2–4 mean slight discoloration of the callus, some browning of the callus, and complete browning of the callus, respectively.

This work demonstrates that reactions of yellow starthistle calli are similar to those seen in the detached-leaf assays (Table 2). Therefore, this method can be used for screening potential toxins. The method involving callus is preferable to the use of detached leaves for several reasons. First, callus provides a material that is more consistent in nature and is completely free from any secondary organisms that may interfere with the test. This results in a more accurate and reliable screening technique. Second, once the callus has been initiated, it takes less time and space to maintain the cultures than whole plants. Whole plants must be watered continually, take up more space in a growth chamber or greenhouse, and are susceptible to insect pests and pathogens.

The isolate of *P. exigua* used in this study was isolated from and proven to be pathogenic to YST (Widmer, 2003). The *Penicillium* sp. used was isolated from yellow starthistle plants but tests showed it was not pathogenic (data not shown). Using the yellow starthistle calli to screen potential pathogens gave the clearest results between 24 and 48 h after inoculation with the fungal spores (Table 4). The calli exposed to *P. exigua* had a higher rating ($P < 0.0001$) than the calli exposed to the *Penicillium* sp. and the control. At 96 h, the visual distinction between the pathogen, *P. exigua* and the nonpathogen *Penicillium* sp. were not obvious. This may be due partially to the obscurity of fungal growth within the liquid medium. However, these results show that it would be possible to screen pathogenic fungi using this technique.

Yellow starthistle is a noxious weed that has been difficult to manage requiring integrated strategies (Sheley et al., 1999) Exploration and research is being conducted to discover new plant pathogens for use as biocontrol agents against this weed. With the techniques presented above for production of yellow starthistle callus and its use for screening potential fungi and toxins, the initial step in the discovery of new biological control agents can proceed faster. This is the first reported attempt at the production of YST callus for screening purposes.

Table 4
Comparison of the pathogenic *Phoma exigua* and nonpathogenic *Penicillium* sp. on *Centaurea solstitialis* calli

Treatment	Average callus rating[a]		
	24 h[b]	48 h	96 h
Control	1.0 ± 0.0b	1.0 ± 0.0b	1.4 ± 0.5c
Phoma exigua	2.4 ± 0.9a	3.2 ± 1.1a	3.9 ± 1.0a
Penicillium sp.	1.3 ± 0.5b	1.7 ± 0.9b	2.8 ± 0.9b

[a] Average rating of *C. solstitialis* calli over time based on a scale of 1 (green, healthy callus) to 5 (complete browning and disintegration of the callus). Ratings of 2–4 mean slight discoloration of the callus, some browning of the callus, and complete browning of the callus, respectively.

[b] Time of incubation at 26 °C. Means followed by the same letter within each column are not significantly different ($P = 0.05$) according to LSD analysis.

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